



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C09B 19/00, C07D 265/34, G01N 33/533		A1	(11) International Publication Number: WO 97/29154
			(43) International Publication Date: 14 August 1997 (14.08.97)
(21) International Application Number: PCT/GB97/00324 (22) International Filing Date: 5 February 1997 (05.02.97) (30) Priority Data: 9602265.2 5 February 1996 (05.02.96) GB (71) Applicant (for all designated States except US): AMERSHAM INTERNATIONAL PLC [GB/GB]; Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SIMMONDS, Adrian [GB/GB]; 12 The Fennings, Amersham, Buckinghamshire HP6 5LE (GB). MILLER, James, N. [GB/GB]; 30 Benscliffe Drive, Loughborough, Leics LE11 3JP (GB). MOODY, Christopher, John [GB/GB]; 9 Kinnerton Way, Exwick, Exeter, Devon EX4 2BL (GB). SWANN, Elizabeth [GB/GB]; 61 Clonkeen Road, Randalstown, County Antrim BT41 3JJ (GB). BRIGGS, Mark, Samuel, Jonathan [GB/GB]; 109 Park Place, Amersham, Buckinghamshire HP6 6NQ (GB). BRUCE, Ian, Edward [GB/IE]; 37 Greenane, Dunslaughin, County Meath (IE). (74) Agent: PENNANT, Pyers; Stevens Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.
(54) Title: BENZOPHENOXAZINE DYES (57) Abstract Benzophenoxazine compounds have formula (I) where X is O or NH, Y is NR ¹ R ² or H, R ¹ and R ² are alkyl or -L-A, L is a linker and A may be a reactive group by means of which the compound is linked to a biomolecule. The compounds can be used as fluorescent dyes for labelling biomolecules.			
<div style="text-align: center;"> </div> <div style="text-align: right;">(I)</div>			

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BENZOPHENOXAZINE DYES

5 Benzophenoxazine compounds provide a family of
fluorescent dyes which can be used for labelling biological molecules in
various applications. The phenoxazine dyes tend to have large Stokes'
shifts of 80 - 100 nm. This is useful in simplifying the type of filtering
needed to differentiate between excitation and emission wavelengths, and
10 may enable sensitivity limits to be improved. The benzophenoxazines also
appear to have particularly good photostability properties.

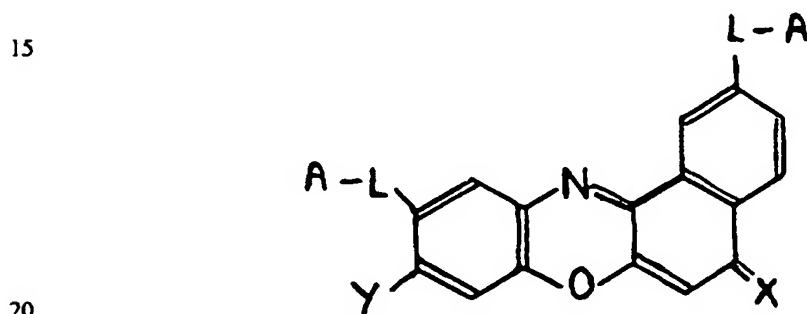
 The benzophenoxazine dye Nile Red (9-
diethylaminobenzo[a]phenoxazine-5-one) (Fig 1) has been shown to be
useful in the fluorescence detection of proteins. Binding of dye to protein
15 has generally been accomplished through non-covalent, hydrophobic
interactions. Fluorescence properties of the dye such as emission
wavelength and intensity have been shown to be dependent on the polarity
of the environment and this appears to be the reason for the different
spectral properties on binding to different proteins. Its low fluorescence in
20 water is thought to be due, at least in part, to the formation of dimers and
higher aggregates, since there is a time-dependent decrease in intensity
on addition to water.

 In order to allow specific labelling of proteins and other
biomolecules such as nucleic acids, attempts have been made to form
25 covalent links, e.g. through the nitrogen at C5 on Nile Blue
(5-amino-9-diethylaminobenzo[a]phenoxazine) (Fig. 1). Linkage to Nile
Blue in this way, however, has been shown to be unsatisfactory because of
the adverse effect this modification has on the dye's fluorescence
properties (Mank *et al*, 1995. Anal. Chem., 67, 1742). Fluorescence
30 quantum yield is reduced by a factor of 10 (from 27 to 2.50) and extinction

coefficient is reduced by almost 20 times (from 75000 to 4000) (Karnes *et al*, 1995, Proc. SPIE-Int. Soc. Opt. Eng., 2388, 21).

The benzophenoxazine ring numbering scheme is also shown in Figure 1. This invention is concerned with novel derivatives of benzophenoxazines which have substituents at the 2 and/or 10 and/or the 9 position, which substituents do not cause a significant loss of fluorescence. Reactive groups at all three positions can be used for linkage to tracer molecules such as biomolecules or to introduce water solubilising functions in order to improve compatibility with aqueous systems or to change the fluorescence emission wavelength of the compound.

According to this invention there is provided a benzophenoxazine compound having the formula



where X is O or NH or N-alkyl or N-aryl or N-alkenyl,

Y is NR^1R^2 or H,

R^1 and R^2 are the same or different and each is C1 - C12 aryl, alkenyl or alkyl or is -L-A,

in at least one -L-A:

- each L is a linker chain of 0 - 20 carbon atoms and which may contain one or more O or N or S atoms, and

each A may be amine or amide or -CN or alcohol or thiol or carboxyl or sulfonate or phosphate, or a reactive group by means of which

30

the benzophenoxazine compound may be covalently linked to a biomolecule or a group which enhances or reduces water solubility or provides electron donor or withdrawal properties to modify the spectral characteristics of the dye,

5 and each other -L-A is H or C1 - C20 alkyl.

In these compounds, the 5-substituent X may be O as in Nile Red or NH as in Nile Blue, or may be N-alkyl, N-aryl or N-alkenyl as in related dyes such as oxazine 170.

At the 9-position, each N-substituent R¹ and R² is the same or
10 different C1 - C12 alkyl or aryl or alkenyl or is -L-A.

In at least one -L-A group, L is a linker chain of 0 to 20 carbon atoms and which may include O or N or S atoms. Preferably the chain is at least three atoms in length, so as to avoid possible steric inhibition of reactions, e.g. -OC₅H₁₀- and -OCOC₄H₈- and -C₂H₄CO-.

15 The compound contains at least one group A which is an amine or amide or -CN or alcohol or thiol or carboxyl or sulfonate or phosphate or a reactive group by means of which the benzophenoxazine compound may be covalently linked to a biomolecule, or a group which enhances (e.g. polyether) or reduces (e.g. alkyl) water solubility or provides
20 electron donor or withdrawal properties to modify the spectral characteristics of the dye, e.g. halogen, alkoxy, nitro or cyano. See The Chemistry of Synthetic Dyes, Venkataraman, Academic Press, N.Y. 1971, 4, Chapter 5 part iiic, p 228-240, particularly Table 1 on page 230. Preferred biomolecules are nucleosides, nucleotides and analogues
25 thereof, oligonucleotides and nucleic acids, and also peptides, proteins, polysaccharides, lipids, sugars and other small molecules.

In another aspect, the invention provides a complex of a benzophenoxazine compound as discussed and a biomolecule, wherein the benzophenoxazine compound is covalently linked to the biomolecule
30 through a reactive group A. These linkages can be conventional. For

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example, a carboxylate group A may be converted to a succinimide ester for reaction with an amine group of a nucleoside or nucleotide or peptide. Examples of other reactive groups most commonly used are isothiocyanate, iodoacetamides, sulphonyl chlorides, maleimides, 5 phosphoramidites, phosphonates and azides. Preferably the linkage is through the 2-position, or through the 9-position or the 10-position of the benzophenoxazine molecule.

Any other group -L-A may optionally be hydrogen or C1 - C20 alkyl.

10

EXPERIMENTAL

Commercially available solvents and reagents were used throughout without further purification, except for those detailed below which were purified as described. DMF was dried by stirring over calcium 15 hydride for 15h, decanted and distilled under reduced pressure before storage over molecular sieves under nitrogen. Methanol was distilled from magnesium turnings and iodine.

Analytical thin layer chromatography was carried out using aluminium-backed plates coated with Merck Kieselgel 60 GF₂₅₄. Plates 20 were visualised under UV light (at 254 and/or 360 nm). Flash chromatography was carried out using Merck Kieselgel 60 H silica or Matrex silica 60. Pressure was applied at the column head with hand bellows. Samples were applied pre-adsorbed on silica. HPLC purification was carried out using either a C18 reverse phase column equilibrated in 25 water with methanol elution or a PRP1 column equilibrated with 2% ammonia and using methanol elution.

IR spectra were recorded in the range 4000-600 cm⁻¹ using a Nicolet FT-205 spectrometer, with internal calibration. Spectra were recorded as KBr discs. UV/visible spectra were obtained using a 30 Shimadzu UV-160 spectrophotometer. Uncorrected fluorescence spectra

were recorded as dilute solutions on a Perkin Elmer LS50B luminescence spectrometer or a Hitachi F4500 using optiglass fluorescence cells.

¹H and ¹³C NMR spectra were recorded on HPLC purified samples using Bruker AC-250, Bruker DPX-400 and Bruker WH-400 instruments (SERC
5 NMR Spectroscopy Centre, Warwick, UK) and a Jeol GSX-270 instrument; J values were recorded in Hz. High and low-resolution mass spectra were recorded on a Kratos MS80 instrument or on a VG Analytical ZAB-E instrument (SERC Mass Spectrometry Service, Swansea). Melting points were measured on an Electrothermal digital melting point apparatus or on
10 a Reichert-Kofler hot stage apparatus and are uncorrected.

Example 1

Introduction of functional groups at the 2 position

15 9-Diethylamino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (I)

5-Diethylamino-2-nitrosophenol hydrochloride (1.14 g, 4.96 mmol) and 1,6-dihydroxynaphthalene (0.19 g, 4.94 mmol) were refluxed in DMF (100 ml) for 4 h. The DMF was removed under reduced pressure. The crude mixture was purified by flash chromatography (ethyl
20 acetate - 1:1 ethyl acetate : isopropanol elution), to yield a dark green crystalline solid.

Yield (1.1 g, 65%)

m.p. >300°C

(Found: M⁺ 334.1317. C₂₀H₁₈N₂O₃ requires 334.1317);

25 ν_{\max} (KBr)/cm⁻¹ 3375, 2964, 1645, 1590, 1561;

λ_{\max} (MeOH)/nm 547 (ϵ 30366), 326 (5762), 265 (27555),

210 (23380);

δ_{H} (400MHz; DMSO *d* 6) 10.42 (1H, broad s, OH), 7.96 (1H, d, *J*=8.6 Hz, 4-H), 7.87 (1H, d, *J*=2.4 Hz, 1-H), 7.55 (1H, d, *J*=9.0 Hz, 11-H), 7.08 (1H, dd, *J*=8.6 Hz, *J*=2.5 Hz, 3-H), 6.77 (1H, dd, *J*=9.0 Hz, *J*=2.5
30 H),

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Hz, 10-H), 6.61 (1H, d, $J=2.5$ Hz, 8-H), 6.13 (1H, s, 6-H), 3.48 (4H, q, $J=7.0$ Hz, $N(CH_2CH_3)_2$), 1.15 (6H, t, $J=7.0$ Hz, $N(CH_2CH_3)_2$);

δ_C (100.6 MHz; DMSO d_6) 181.64 (CO), 160.69, 151.66, 150.74, 146.47, 138.77, 133.82, 130.88 (CH), 127.52 (CH), 123.92, 118.42 (CH), 109.96 (CH), 108.18 (CH), 104.15 (CH), 96.09 (CH), 44.48 (CH_2CH_3), 12.52 (CH_2CH_3);

m/z (EI) 335 (MH^+ , 20%), 334 (M^+ , 63), 320 (25), 319 (100), 291 (26); fluorescence in methanol strong.

10 **9-Diethylamino-5-oxo-5H-benzo[a]phenoxazine-2-carboxylic acid (II)**

This was made similarly to the hydroxy derivative above except that the starting materials were 5-diethylamino-2-nitrosophenol hydrochloride and 1-hydroxynaphthalene-6-carboxylic acid.

15 **Example 2**

Derivatisation of 2-hydroxyl analogue with a 6 carbon linker through ester formation

20 **9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yl (5-carboxy)pentanoate (III)**

A mixture of the 9-diethylamino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (0.096 g, 0.28 mmol), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (0.18 g, 0.42 mmol), adipic acid (0.046 g, 0.31 mmol) and a catalytic quantity of 4-dimethylaminopyridine were stirred overnight in dichloromethane (30 ml). The crude mixture was washed with water, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (ethyl acetate elution) to yield the acid as a dark red solid.

Yield (0.08 g, 60 %)

30

m.p. 85-87°C

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$\nu_{\max}(\text{film})/\text{cm}^{-1}$ 3347, 3055, 2985, 2930, 1757, 1639, 1586

$\lambda_{\max}(\text{MeOH})/\text{nm}$ 558 (ϵ 22222), 267 (28090), 208 (17094)

$\delta_{\text{H}}(250 \text{ MHz}; \text{CDCl}_3)$ 8.32 (2H, 4-H, 1-H), 7.56 (1H, d,

$J=9.1 \text{ Hz}$, 11-H), 7.34 (1H, dd, $J=8.7 \text{ Hz}$, $J=2.3 \text{ Hz}$, 3-H), 6.62 (1H, dd,

5 $J=9.2 \text{ Hz}$, $J=2.7 \text{ Hz}$, 10-H), 6.44 (1H, d, $J=2.7 \text{ Hz}$, 8-H), 6.40 (1H, s, 6-H),

3.47 (4H, q, $J=7.1 \text{ Hz}$, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 2.68 (2H, t, $J=7.0 \text{ Hz}$, COCH_2), 2.49

(2H, t, $J=6.9 \text{ Hz}$, COCH_2), 1.26 (6H, t, $J=7.1 \text{ Hz}$, $\text{N}(\text{CH}_2\text{CH}_3)_2$).

$\delta_{\text{H}}(100.6 \text{ MHz}; \text{CDCl}_3)$ 182.91 (CO), 171.41 (CO), 153.18,

152.41, 151.05, 146.94, 139.05, 133.72, 131.30 (CH), 129.46, 127.66

10 (CH), 124.99, 123.48 (CH), 116.36 (CH), 109.93 (CH), 105.50 (CH), 96.39

(CH), 45.14 (CH_2CH_3), 34.05 (CH_2), 29.70 (CH_2), 24.24 (CH_2), 24.14 (CH_2),

12.62 (CH_2CH_3)

m/z (CI) 463 (MH^+ , 84%), 335 (100), 146 (13), 74 (18)

fluorescence in methanol strong

15

Example 3

Derivatisation of 2-hydroxyl analogue with a 6 carbon linker through ether formation

20 **Benzyl 6-(9-diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate (IV)**

A mixture of 9-diethylamino-2-hydroxy-5H-

benzo[a]phenoxazin-5-one (0.1 g, 0.3 mmol), potassium carbonate (0.12 g,

0.9 mmol) and benzyl 6-bromohexanoate (0.11 g, 0.38 mmol) in DMF (50

25 ml) was refluxed for 4 h. Residual potassium carbonate was filtered off

and the filtrate concentrated under reduced pressure. The crude residue

was purified by column chromatography (petrol -petrol: ether, 1:1, 10%

gradient elution) to yield a green crystalline solid.

m.p. 146-148°C

30

(Found: M^+ 538.2470. $\text{C}_{33}\text{H}_{34}\text{N}_2\text{O}_5$ requires 538.2467);

ν_{\max} (KBr)/cm⁻¹ 2937, 2865, 1734, 1595;

λ_{\max} (MeOH)/nm 551 (ϵ 27169), 267 (28144), 210 (30531);

δ_{H} (250 MHz; CDCl₃) 8.20 (1H, d, J =8.7 Hz, 4-H), 8.02 (1H, d, J =2.5 Hz, 1-H), 7.59 (1H, d, J =9.1 Hz, 11-H), 7.35 (5H, broad s, Ph),
 5 7.13 (1H, dd, J =8.7 Hz, J =2.6 Hz, 3-H), 6.63 (1H, dd, J =9.1 Hz, J =2.6 Hz, 10-H), 6.44 (1H, d, J =2.6 Hz, 8-H), 6.28 (1H, s, 6-H), 5.13 (2H, s, CH₂Ph),
 4.14 (2H, t, J =6.2 Hz, ArOCH₂), 3.45 (4H, q, J =7.0 Hz, N(CH₂CH₃)₂), 2.43
 (2H, t, J =7.3 Hz, CH₂CO₂Bn), 1.85 (2H, m), 1.76 (2H, m), 1.58 (2H, m),
 1.25 (6H, t, J =7.0 Hz, N(CH₂CH₃)₂);

10 δ_{C} (100.6 MHz; CDCl₃) 184.14 (CO), 1173.34 (CO₂Bn),
 161.60, 151.90, 150.59, 146.67, 139.76, 133.95, 130.95 (CH), 128.50
 (CH), 128.14 (CH), 127.56 (CH), 125.44, 124.57, 118.13 (CH), 109.40
 (CH), 106.39 (CH), 105.10 (CH), 96.12 (CH), 67.93 (CH₂), 66.09 (CH₂),
 44.97 (N(CH₂CH₃)₂), 34.14 (CH₂), 28.82 (CH₂), 25.59 (CH₂), 24.64
 15 (CH₂), 12.57 (N(CH₂CH₃)₂)

m/z (CI) 539 (MH⁺, 46%), 335 (22), 165 (22), 132 (100);

fluorescence in methanol strong

Methyl 6-(9-diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate (V)
 20

This was formed by transesterification from the benzyl ester using the same procedure described for formation of the benzyl ester (IV) but using methanol as solvent.

m.p. 152-154°C

25 ν_{\max} (KBr)/cm⁻¹ 2929, 2868, 1739, 1620, 1601, 1581;
 λ_{\max} (MeOH)/nm 553 (ϵ 27800), 265 (27433), 212 (21459);
 δ_{H} (400 MHz; CDCl₃) 8.19 (1H, d, J =8.7 Hz, 4-H), 8.01 (1H, d, J =2.6 Hz, 1-H), 7.57 (1H, d, J =9.0 Hz, 11-H), 7.13 (1H, dd, J =8.7 Hz, J =2.6 Hz, 3-H), 6.62 (1H, dd, J =9.1 Hz, J =2.7 Hz, 10-H), 6.42 (1H, d,

$J=2.7$ Hz, H₈), 6.26 (1H, s, 6-H), 4.16 (2H, t, $J=6.4$ Hz, ArOCH₂), 3.68 (3H, s, CO₂Me), 3.44 (4H, q, $J=7.1$ Hz, N(CH₂CH₃)₂), 2.38 (2H, t, $J=7.4$ Hz, CH₂CO₂Me), 1.88 (2H, m), 1.74 (2H, m), 1.58 (2H, m), 1.25 (6H, t, $J=7.1$ Hz, v (CH₂CH₃)₂);

5 δ_C (100.6 MHz; CDCl₃) 183.51 (CO), 174.30 (CO), 152.30, 150.98, 147.09, 140.33, 134.33, 131.321, 128.83 (CH), 125.88 (CH), 124.95, 122.15, 117.61 (CH), 109.74 (CH), 106.88 (CH), 105.56 (CH), 96.58 (CH), 68.38 (CH₂), 51.78 (CO₂Me), 44.98 (CH₂), 34.26 (CH₂), 29.18 (CH₂), 25.97 (CH₂), 24.98 (NCH₂CH₃), 12.90 (NCH₂CH₃);

10 m/z (ES) 463 (MH⁺, 28%), 234 (14), 194 (28), 179 (37), 98 (67), 84 (100); fluorescence in methanol strong

Ethyl 6-(9-diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate (VI)

15 A mixture of 9-diethylamino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (0.27 g, 0.81 mmol), potassium carbonate (0.34 g, 2.46 mmol) and ethyl 6-bromohexanoate (0.28 g, 1.26 mmol) in methanol (100 ml) was refluxed for 2 days. After this time full conversion was observed by TLC. Excess potassium carbonate was filtered off and
20 the filtrate concentrated. The crude residue was evaporated and purified by column chromatography (ether elution), to yield a red crystalline solid.

Yield (0.21 g, 53%)

m.p. 158-160°C

(Found: M^+ 476.2311. C₂₈H₃₂N₂O₅ requires 476.2311);

25 ν_{\max} (KBr)/cm⁻¹ 2927, 1735, 1628, 1600, 1582;

λ_{\max} (MeOH)/nm 552 (ϵ 44064), 267 (40664), 208 (38216);

δ_H (250MHz; CDCl₃) 8.21 (1H, d, $J=8.7$ Hz, 4-H), 8.04 (1H, d, $J=2.6$ Hz, 1-H), 7.60 (1H, d, $J=9.1$ Hz, 11-H), 7.15 (1H, dd, $J=8.7$ Hz, $J=2.5$ Hz, 3-H), 6.65 (1H, dd, $J=9.1$ Hz, $J=2.7$ Hz, 10-H), 6.45 (1H, d, $J=2.7$

Hz, 8-H), 6.29 (1H, s, 6-H), 4.15 (4H, m, CO₂CH₂CH₃, ArOCH₂), 3.47 (4H, q, *J*=7.1 Hz, N(CH₂CH₃)₂), 2.36 (2H, t, *J*=7.2 Hz, CH₂CO₂Et), 1.89 (2H, m), 1.75 (2H, m), 1.57 (2H, m), 1.26 (9H, t, *J*=7.1 Hz, N(CH₂CH₃)₂, CO₂CH₂CH₃);

- 5 δ_C (62.9 MHz; CDCl₃) 183.14 (CO), 173.86 (CO₂Et), 161.63, 151.92, 150.60, 146.69, 140.00, 133.95, 130.94 (CH), 127.57 (CH), 125.46, 124.57, 118.14, 109.39 (CH), 106.42 (CH), 105.13 (CH), 96.15 (CH), 67.95 (CH₂), 60.20 (CH₂), 44.97 (CH₂), 34.18 (CH₂), 28.84 (CH₂), 25.61 (CH₂), 24.65 (CH₂), 14.19 (CH₃), 12.55 (CH₃);
- 10 *m/z* (EI) 477 (MH⁺, 15%), 476 (M⁺, 67), 461 (73), 431 (13), 291 (15), 233 (17); fluorescence in methanol strong.

6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoic acid (VII)

- 15 The ethyl ester (ethyl 6-(9-diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate) (0.055 g, 0.11 mmol) was suspended in ammonium dihydrogen phosphate (0.1 M, 5 ml, pH 7) and porcine liver esterase (0.6 ml, 1500 units) added. The mixture was stirred at 37°C for 7 days and checked by TLC. The water was removed under
- 20 reduced pressure. The residue was dissolved in methanol and the enzyme extract filtered off through celite. The crude mixture was purified by reverse phase chromatography (methanol elution), to yield a dark red crystalline solid.

- Yield (0.032 g, 62%)
- 25 m.p. 167-169°C
- (Found: MH⁺449.2076. C₂₆H₂₈N₂O₅ + H requires 449.2076);
- ν_{\max} (KBr)/cm⁻¹ 3435, 2953, 1653, 1638, 1619, 1593;
- λ_{\max} (MeOH)/nm 550 (ϵ 30423), 325 (5824), 267 (28753).

210 (20759);

δ_H (250MHz; MeOH *d* 4) 8.02 (1H, d, *J*=8.8 Hz, 4-H), 7.92 (1H, d, *J*=2.4 Hz, 1-H), 7.52 (1H, d, *J*=9.2 Hz, 11-H), 7.10 (1H, dd, *J*=8.8 Hz, *J*=2.5 Hz, 3-H), 6.76 (1H, dd, *J*=9.2 Hz, *J*=2.5 Hz, 10-H), 6.51 (1H, d, *J*=2.5 Hz, 8-H), 6.14 (1H, s, 6-H), 4.09 (2H, t, *J*=7.7 Hz, ArOCH₂), 3.49 (4H, q, *J*=7.1 Hz, N(CH₂CH₃)₂), 2.33 (2H, t, *J*=7.1 Hz, CH₂CO₂H), 1.86 (2H, m), 1.69 (2H, m), 1.56 (2H, m), 1.24 (6H, t, *J*=7.1 Hz, N(CH₂CH₃)₂);

δ_C (100.6 MHz; DMSO *d* 6) 181.49 (CO), 174.77 (CO₂H), 161.34, 151.78, 150.88, 146.54, 138.33, 133.64, 131.05 (CH), 127.30 (CH), 124.91, 124.00, 117.98 (CH), 110.12 (CH), 106.25 (CH), 104.14 (CH), 96.04 (CH), 67.95 (CH₂), 44.54 (CH₂), 34.01 (CH₂), 28.50 (CH₂), 25.26 (CH₂), 24.51 (CH₂), 12.57 (CH₃);

m/z (FAB) 449 (MH⁺, 54%), 330 (42), 308 (73), 290 (56), 165 (65); fluorescence in methanol strong

15

Example 4

Labelling of model biological molecules

Synthesis of the active ester (succinyl 6-(9-diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate) (VIII)

6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoic acid (0.12g, 0.26 mmol), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (0.34 g, 0.8 mmol), N-hydroxysuccinimide (0.09 g, 0.8 mmol) and a catalytic quantity of 4-dimethylaminopyridine were stirred overnight in dichloromethane (25 ml). The solvent was removed under reduced pressure and the residue purified by column chromatography (ether) elution) to yield the active ester as a dark red solid.

Yield (0.13, 92%)

m.p. 166-168°C

(Found: M^+ 545.2160. $C_{30}H_{31}N_3O_7$ requires 545.2162);

ν_{\max} (KBr)/ cm^{-1} 3400, 1737, 1623, 1617, 1589;

5 λ_{\max} (MeOH/nm) 552 (ϵ 30929), 325 (5359), 268 (27886),
210 (34608);

δ_H (250 MHz; CDCl_3) 8.20 (1H, d, $J=8.7$ Hz, 4-H), 8.02 (1H,
d, $J=2.5$ Hz, 1-H) 7.58 (1H, d, $J=9.2$ Hz, 11-H), 7.15 (1H, dd, $J=8.7$ Hz,
 $J=2.5$ Hz, 3-H), 6.63 (1H, dd, $J=9.2$ Hz, $J=2.7$ Hz, 10-H), 6.43 (1H, d, $J=2.7$
10 Hz, 8-H), 6.28 (1H, s, 6-H), 4.18 (2H, t, $J=6.2$ Hz, ArOCH_2), 3.45 (4H, q,
 $J=7.1$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 2.84 (4H, s, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.68 (2H, t, $J=7.2$
Hz, $\text{CH}_2\text{CO}_2\text{N}(\text{COCH}_2)_2$), 1.88 (4H, m), 1.68 (2H, m), 1.25 (6H, t, $J=7.1$
Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$);

δ_C (100.6 MHz; CDCl_3) 169.46 (CO), 168.85 (CO), 162.09
15 (CO), 152.42, 151.10, 147.22, 140.50, 134.45 (CH), 131.44 (CH), 128.11,
126.03, 125.09, 118.68 (CH), 109.86 (CH), 107.02 (CH), 105.68 (CH),
96.72 (CH), 68.28 (CH_2), 45.42 (CH_2), 31.30 (CH_2), 29.12 (CH_2), 25.98
(CH_2), 25.77 (CH_2), 24.78 (CH_2), 13.00 ($\text{N}(\text{CH}_2\text{CH}_3)_2$);

m/z (EI) 546 (MH^+ , 26%), 545 (M^+ , 85), 530 (40), 430 (62),
20 415 (88), 262 (52), 233 (100); fluorescence in methanol strong

Reaction of Active Ester with Allylamine (IX)

(a) Homogeneous reaction

25 To a stirred solution of the active ester VIII (0.039 g, 0.07
mmol) and a catalytic quantity of 4-dimethylaminopyridine in
dichloromethane was added dropwise allylamine (0.026 ml, 0.35 mmol).
The mixture was stirred overnight. The solvent was removed under
reduced pressure and the residue purified by column chromatography

(ethyl acetate elution) to yield the allyl amide as a dark red solid (0.02 g, 59%).

(b) Biphasic Reaction

- 5 To a stirred solution of the active ester (0.06g, 0.11 mmol) in dichloromethane (15 ml) was added a solution of sodium hydrogen carbonate (0.1M, 8 ml) and sodium carbonate (0.1M, 8 ml) containing allyl amine (0.04 ml, 0.54 mmol) and tetrabutylammonium hydrogen sulfate (0.037 g, 0.11 mmol). The mixture was stirred for 2 h and followed by TLC.
- 10 The layers were separated and the dichloromethane layer was dried (MgSO₄) and concentrated to yield the allylamide as a dark red solid (0.37 g, 69%).

6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoic acid tryptophan methyl ester amide (X)

- 15 A solution of the active ester VIII (0.035 g, 0.06 mmol), L-tryptophan methyl ester hydrochloride (0.049 g, 0.19 mmol) and pyridine (0.015 g, 0.19 mmol) in dichloromethane (20 ml) was stirred overnight. The solvent was removed under reduced pressure and the residue purified
- 20 by column chromatography (ethyl acetate elution) to yield the tryptophan amide as a dark red crystalline solid.

Yield (0.027 g, 65%)

m.p. 75-77°C

- ν_{\max} (KBr)/cm⁻¹ 3421, 3300, 2930, 1750, 1735, 1654, 1617,
- 25 1594, 1572;

λ_{\max} (MeOH)/nm 551 (ϵ 38033), 326 (7308), 268 (42555), 220 (70697);

- δ_{H} (250 MHz; CDCl₃) 8.54 (1H, broad s, NH), 8.18 (1H, d, $J=8.7$ Hz, 4-H), 8.00 (1H, $J=2.5$ Hz, 1-H), 7.58 (1H, d, $J=9.1$ Hz, 11-H),
- 30 7.56 (1H, m), 7.36 (1H, m), 7.16 (4H, m), 6.61 (1H, dd, $J=9.1$ Hz, $J=2.7$ Hz,

10-H), 6.43 (1H, d, $J=2.6$ Hz, 8-H), 6.28 (1H, s, 6-H), 6.05 (1H, d, $J=7.8$ Hz, CONH), 4.97 (1H, td, $J=7.8$ Hz, $J=5.6$ Hz, HNCHCO_2Me), 4.04 (2H, t, $J=6.4$ Hz, ArCH_2), 3.71 (3H, s, OMe), 3.45 (4H, q, $J=7.1$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 3.32 (2H, t, $J=5.6$ Hz, $\text{CH}(\text{CO}_2\text{Me})\text{CH}_2$), 2.22 (2H, t, $J=7.4$ Hz, CH_2CON), 1.81 (2H, m), 1.72 (2H, m), 1.63 (2H, m), 1.25 (6H, t, $J=7.1$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$);

δ_{C} (100.6 MHz; CDCl_3) 183.67 (CO), 172.93 (CONH), 162.15 (CO_2Me), 152.46, 151.16, 147.22, 140.35, 136.59, 134.41, 131.38 (CH), 128.12 (CH), 125.92, 125.92, 125.10, 123.18 (CH), 122.60 (CH), 120.01 (CH), 119.88, 119.12, 118.86 (CH), 118.70 (CH), 111.79 (CH), 110.40, 109.97 (CH), 106.94 (CH), 105.61 (CH), 96.70 (CH), 68.42 (CH_2), 53.28 (CHCO_2CH_3), 52.70 (CO_2Me), 45.43 (NCH_2CH_3), 36.78 (CH_2), 29.23 (CH_2), 28.00 (CH_2), 25.92 (CH_2), 25.52 (CH_2), 13.00 ($\text{N}(\text{CH}_2\text{CH}_3)_2$);

15 m/z (ES) 649 (MH^+ , 68%), 489 (9), 348 (23), 263 (100);
fluorescence in methanol strong.

6-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoic acid glycine *tert*-butyl ester amide (XI)

20 A mixture of 6-(9-diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoic acid (10 mg, 22 μmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (ETC) (14 mg, 45 μmol) and N-hydroxysulfosuccinimide sodium salt (15 mg, 67 μmol) in DMF (1 ml) was stirred at room temperature. After 2.5h a further portion of
25 ETC (50 mg, 168 μmol) was added. The mixture was stirred at room temperature overnight and a solution of *tert*-butyl glycine hydrochloride (19 mg, 112 μmol) in pH 9.2 buffer ($\text{NaHCO}_3/\text{Na}_2\text{CO}_3$) (1.5 ml) was added dropwise. After 3h the mixture was concentrated *in vacuo* and purified by flash column chromatography (C18, 50% methanol/water to 80%

methanol/water gradient) to afford the *title compound* as a dark red solid (11 mg, 88%).

δ_H (270 MHz; $CDCl_3$) 8.21 (1H, d, $J=8.8$ Hz, 4-H), 8.03 (1H, d, $J=2.5$ Hz, 1-H), 7.61 (1H, d, $J=9.1$ Hz, 11-H), 7.15 (1H, dd, $J=8.6$ Hz and $J=2.6$ Hz, 3-H), 6.65 (1H, dd, $J=9.3$ Hz and 2.7 Hz, 10-H), 6.45 (1H, d, $J=2.8$ Hz, 8-H), 6.29 (1H, s, 6-H), 6.00 (1H, br t, $J=5.2$ Hz, NH), 4.17 (2H, t, $J=6.3$ Hz, $ArOCH_2$), 3.95 (2H, d, $J=5.2$ Hz, $NHCH_2$), 3.47 (4H, q, $J=7.1$ Hz, $N(CH_2CH_3)_2$), 2.30 (2H, d, $J=7.4$ Hz, CH_2CON), 1.95-1.68 (4H, m), 1.68-1.50 (2H, m), 1.47 (9H, s, Bu^t) and 1.26 (2H, t, $J=7$ Hz, $N(CH_2CH_3)_2$)

10 fluorescence in methanol strong

Example 5

Derivatisation at the 10 position with alkylcarboxy or alkylamino groups

15 10-(2-aminoethyl)-5H-benzo[a]phenoxazin-5-one (XII)

This was synthesised according to the methods of Bhansali and Kook (Petrocycles, 36, 1239-51, 1993).

10-(2-carboxyethyl)-5H-benzo[a]phenoxazin-5-one (XIII)

20 This was synthesised according to the methods of Bhansali and Kook (1993) but using 3-(4-hydroxyphenyl)propionic acid as the phenolic substrate.

Example 6

Introduction of water-solubilising functionality into the dialkylamino grouping at the 9 position

5 **9(N-Ethyl-(N-3-sulfonylpropyl))amino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (XIV)**

3-(N-Ethyl-3-hydroxy-4-nitrosoanilino)-propanesulfonic acid hydrochloride (0.53 g, 1.6 mmol) and 1,6-dihydroxynaphthalene (0.26 g, 1.6 mmol) were refluxed in DMF (40 ml) for 4 h. The DMF was removed
10 under reduced pressure. The crude mixture was purified by flash chromatography (ethyl acetate-1:1 ethyl acetate: methanol, 10% gradient elution) to yield a dark green crystalline solid.

Yield (0.54 g, 79%)

m.p. 147-149°C

15 ν_{max} (KBr)/cm⁻¹ 3421, 2970, 1639, 1617, 1592, 1560;

λ_{max} (MeOH)/nm 547 (ϵ 28969), 326 (5541), 264 (26761),
212 (21805);

δ_{H} (400MHz; MeOH *d* 4) 7.92 (1H, d, *J*=8.6 Hz, 4-H), 7.76 (1H, d, *J*=2.4 Hz, 1-H), 7.35 (1H, d, *J*=9.1 Hz, 11-H), 6.98 (1H, dd, *J*=8.6 Hz, *J*=2.5 Hz, 3-H), 6.68 (1H, dd, *J*=9.1 Hz, *J*=2.5 Hz, 10-H), 6.41 (1H, d, *J*=2.5 Hz, 8-H), 6.03 (1H, s, 6-H), 3.53 (2H, t, *J*=7.7 Hz, HO₃SCH₂CH₂CH₂N), 3.46 (2H, q, *J*=7.0 Hz, CH₃CH₂N), 2.91 (2H, t, *J*=7.2 Hz, HO₃SCH₂), 2.11 (2H, m, HO₃SCH₂CH₂CH₂N), 1.22 (3H, t, *J*=7.0 Hz, CH₃CH₂N);
20

25 δ_{C} (100 MHz; MeOH *d* 4) 182.91 (CO), 160.09, 151.65, 150.80, 145.82, 137.56, 133.52, 130.15 (CH), 126.49 (CH), 124.24, 123.14, 117.02 (CH), 109.73 (CH), 107.60 (CH), 102.64 (CH), 95.28 (CH), 48.47 (CH₂), 47.67 (CH₂), 44.40 (CH₂), 22.21 (CH₂), 10.69 (CH₃);

m/z (ES) 428 (M^- , 23%), 427 ($M-H^-$, 100);

fluorescence in methanol strong.

Ethyl 6-(9(N-ethyl-(N-3-sulfonylpropyl))amino-5-oxo-5H-

5 benzo[a]phenoxazin-2-yloxy)hexanoate (XV)

A mixture of 9(N-ethyl-(N-3-sulfonylpropyl))amino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (0.34 g, 0.86 mmol), potassium carbonate (0.56 g, 4.06 mmol) and ethyl 6-bromohexanoate (0.95 g, 4.26 mmol) in DMF (25 ml) was refluxed overnight. Excess potassium carbonate was
10 filtered off and the filtrate concentrated. The crude residue was purified by column chromatography (ethyl acetate -1:1 ethyl acetate: methanol, 10% gradient elution) to yield a red crystalline solid.

Yield (0.4 g, 87%)

m.p. 150-152°C

15 ν_{\max} (KBr)/ cm^{-1} 3442, 2928, 2869, 1733, 1619, 1596;

λ_{\max} (MeOH)/nm 552 (ϵ 20732), 326 (3963), 266 (19539),
212 (14886);

δ_H (400MHz; MeOH d_4) 7.93 (1H, d, $J=8.6$ Hz, 4-H), 7.81
(1H, d, $J=2.5$ Hz, 1-H), 7.44 (1H, d, $J=9.1$ Hz, 11-H), 7.00 (1H, dd, $J=8.7$
20 Hz, $J=2.5$ Hz, 3-H), 6.76 (1H, dd, $J=9.3$ Hz, $J=2.5$ Hz, 10-H), 6.50 (1H, d,
 $J=2.5$ Hz, 8-H), 6.05 (1H, s, 6-H), 4.04 (4H, m, ArOCH_2 , $\text{CO}_2\text{CH}_2\text{CH}_3$),
3.50 (4H, m, $\text{HO}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{N}$, NCH_2CH_3), 2.82 (2H, t, $J=7.2$ Hz,
 $\text{HO}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.29 (2H, t, $J=7.3$ Hz, $\text{CH}_2\text{CO}_2\text{Et}$), 2.03 (2H, m,
 $\text{HO}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{N}$), 1.77 (2H, m), 1.62 (2H, m), 1.47 (2H, m), 1.16 (6H,
25 m, NCH_2CH_3 , $\text{CO}_2\text{CH}_2\text{CH}_3$);

δ_C (100.6 MHz; MeOH d_4) 184.18 (CO), 174.70 (CO_2Et),
162.54, 153.12, 152.29, 147.35, 138.87, 134.64, 131.58 (CH), 127.60
(CH), 125.55, 125.35, 118.41 (CH), 111.20 (CH), 106.65 (CH), 96.67 (CH).
68.50 (CH_2), 60.66 (CH_2), 49.26 (CH_2), 45.72 (CH_2), 34.26 (CH_2), 29.14

(CH₂), 25.03 (CH₂), 23.44 (CH₂), 13.78 (CH₃), 11.94 (CH₃);

m/z (ES) 570 (M⁺, 39%), 569 (M-H⁺, 100);

fluorescence in methanol strong

5 **Benzyl 6-(9-(N-ethyl-(N-3-sulfonylpropyl))amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate (XVI)**

A mixture of 9(N-ethyl-(N-3-sulfonylpropyl))amino-2-hydroxy-5H-benzo[a]phenoxazin-5-one (0.54 g, 1.26 mmol), potassium carbonate (0.52 g, 3.76 mmol) and benzyl 6-bromohexanoate (0.47 g, 1.65 mmol) in
10 DMF (25 ml) was refluxed overnight. Excess potassium carbonate was filtered off and the filtrate concentrated. The crude residue was purified by column chromatography (ethyl acetate -1:1 ethyl acetate: methanol, 10% gradient elution) to yield a red crystalline solid.

Yield (0.4 g, 51%)

15 m.p. 156-158°C

ν_{\max} (KBr)/cm⁻¹ 3448, 2938, 1735, 1640, 1619, 1595;

λ_{\max} (MeOH)/nm 551 (ϵ 7973), 326 (1546), 267 (7535), 209 (7496);

δ_{H} (400 MHz; MeOH *d* 4) 7.98 (1H, d, *J*=8.7 Hz, 4-H), 7.83
20 (1H, d, *J*=2.4 Hz, 1-H), 7.48 (1H, d, *J*=9.1 Hz, 11-H), 7.30 (5H, m, Ph), 7.03 (1H, dd, *J*=8.8 Hz, *J*=2.5 Hz, 3-H), 6.80 (1H, dd, *J*=9.1 Hz, *J*=2.6 Hz, 10-H), 6.54 (1H, d, *J*=2.6 Hz, 8-H), 6.10 (1H, s, 6-H), 5.12 (2H, s, CH₂Ph), 4.03 (2H, t, *J*=6.4 Hz, ArOCH₂), 3.54 (2H, t, *J*=7.7 Hz, HO₃SCH₂CH₂CH₂N), 3.51 (2H, q, *J*=7.1 Hz, NCH₂CH₃), 2.91 (2H, t, *J*=7.2 Hz, HO₃SCH₂), 2.44
25 (2H, t, *J*=7.3 Hz, CH₂OBn), 2.14 (2H, m, HO₃CH₂CH₂CH₂N), 1.80 (2H, m), 1.74 (2H, m), 1.52 (2H, m), 1.25 (3H, t, *J*=7.1 Hz, NCH₂CH₃);

δ_{C} (100.6 MHz; MeOH *d* 4) 184.97 (CO), 175.19 (CO₂Bn), 163.28, 153.85, 153.08, 148.11, 139.66, 137.73, 135.39, 132.37, 129.58 (CH), 129.23 (CH), 128.40 (CH), 126.35, 119.20 (CH), 111.98 (CH),

107.47 (CH), 97.48 (CH), 69.28 (CH₂), 67.23 (CH₂), 46.54 (CH₂), 35.07 (CH₂), 26.65 (CH₂), 25.90 (CH₂), 25.87 (CH₂), 24.30 (CH₂), 12.80 (CH₃);

m/z (ES) 633 (MH⁺, 17%), 632 (M⁺, 43), 281 (30), 255 (49), 177 (60), 130 (68), 100 (100); fluorescence in methanol strong.

5

6-(9-N-Ethyl-(3-sulfonylpropyl)amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoic acid (XVII)

Ethyl 6-(9(N-ethyl-(N-3-sulfonylpropyl))amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)hexanoate (0.3 g, 0.55 mmol) was dissolved
10 in ammonium dihydrogen phosphate (0.1 M, 50 ml. pH 7) and porcine liver esterase (6.3 ml, 20000 units) added. The mixture was stirred at 37°C for 14 days. The water was removed under reduced pressure. The residue was dissolved in methanol and the enzyme extract filtered off through celite. The crude mixture was purified by reverse phase chromatography
15 on preadsorbed silica (methanol elution), to yield a dark red crystalline solid.

Yield (0.15 g, 53%)

m.p. 222-225°C

*v*_{max} (KBr)/cm⁻¹ 3441, 3064, 2934, 1732, 1640, 1619, 1594;

20 *λ*_{max} (MeOH)/nm 557 (ε 28648), 531 (31100), 325 (6233), 261 (28726), 216 (21551);

*δ*_H (250 MHz; MeOH *d* 4) 7.74 (1H, d, *J*=8.8 Hz, 4-H), 7.49 (1H, d, *J*=2.3 Hz, 1-H), 7.22 (1H, d, *J*=9.1 Hz, 11-H), 6.82 (1H, dd, *J*=8.7 Hz, *J*=2.5 Hz, 3-H), 6.65 (1H, dd, *J*=9.1 Hz, *J*=2.3 Hz, 10-H), 6.31 (1H, d, *J*=2.3 Hz, 8-H), 5.86 (1H, s, 6-H), 3.89 (2H, t, *J*=6.2 Hz, ArOCH₂), 3.50
25 (4H, m, HO₃SCH₂CH₂CH₂N, NCH₂CH₃), 7.10 (2H, t, *J*=7.1 Hz, HO₃SCH₂), 2.37 (2H, t, *J*=7.2 Hz, CH₂CO₂H), 2.10 (2H, m, HO₃SCH₂CH₂CH₂N), 1.77 (4H, m), 1.50 (2H, m), 1.22 (3H, t, *J*=7.0 Hz, NCH₂CH₃);

δC (100.6 MHz; MeOH *d* 4) 185.21 (CO), 177.96 (CO₂H),
163.71, 154.21, 153.57, 139.93, 135.77, 132.84 (CH), 128.77 (CH),
126.89, 126.47, 119.65 (CH), 112.52 (CH), 107.79 (CH), 105.32 (CH),
97.92 (CH), 69.78 (CH₂), 51.10 (CH₂), 49.07 (CH₂), 47.03 (CH₂), 35.41
5 (CH₂), 30.49 (CH₂), 27.24 (CH₂), 26.39 (CH₂), 24.79 (CH₂), 13.28
(NCH₂CH₃);

m/z (ES) 542 (M⁺, 22%), 255 (14), 177 (100);

fluorescence in methanol strong

10 **Example 7**

Fluorescence characterisation of derivatised molecules.

Effect on fluorescence of derivatisation and linkage of benzophenoxazines to biological molecules.

15 Fluorescence spectra (uncorrected) were determined on 1 μ M
methanol solutions of HPLC purified samples using a Hitachi F4500
spectrofluorimeter. Relative fluorescence was determined from the
emission curve at the peak excitation wavelength using the "area" function
on the fluorimeter.

20

	Excitation peak (nm)	Emission peak (nm)	Relative peak fluorescence
Nile Red	558	635	1
VII	558	633	0.70
XI	558	633	0.72
XVII	558	633	0.73

The data clearly show that modification at the 2 and the 9
position resulted in only a small decrease in fluorescence intensity
compared to the that of the parent compound.

Relative fluorescence in aqueous environment of Nile Red and a derivative containing water solubilising functions.

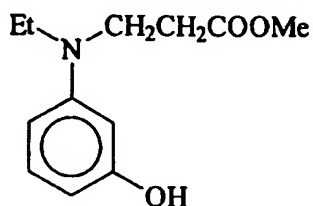
Spectra were determined 1 hour after diluting 100 μ M methanolic solutions 100 fold into 50mM Tris buffer pH 7.1. Relative
5 fluorescence was determined from the emission curve at the peak excitation wavelength using the "area" function on the fluorimeter.

The results showed a time dependent decrease in fluorescence of Nile Red in buffer. After 1 hour there was a 33 fold difference in Nile Red fluorescence intensity in methanol compared to that
10 in water (see table). This was associated with a large light scatter peak at the excitation wavelength, presumably due to aggregation of the fluorophore. Compound XVII, which has a sulfonic acid group to provide water solubilising properties, showed a fluorescence in water which was reduced by only 3.6 fold compared to its fluorescence in methanol. The
15 intensity appeared not to vary with time.

	Peak excitation and emission in methanol (nm)	Relative fluorescence in methanol *	Peak excitation and emission in buffer (nm)	Relative fluorescence in buffer *
Nile Red	558/635	1.00	599/657	0.03
XVII	558/633	0.73	592/651	0.20

* Relative to the fluorescence intensity of Nile Red in methanol.

20 Normalised spectra of Nile Red and compound XI in methanol, and compound XVII in buffer are shown in Figure 3.

Example 8**Synthesis of 9-(N-2-carboxyethyl-N-ethyl)amino-5H-benzo[a]phenoxazin-5-one (XVIII)****5 Synthesis of N-(2-methoxycarbonyl)ethyl-N-ethyl-m-aminophenol****Step 1.****10 Synthesis of N-ethyl-m-aminophenol from resorcinol.**

Resorcinol (200g, 1.82 mol) was heated to 140-150°C and stirred. Monoethylamine* was steadily passed through the melt for 32 hours. The viscous oily product was stirred and washed with hot water (3x 200ml) to remove unreacted resorcinol.

15 The mono-ethylated product was purified by vacuum distillation (bp 159-162°C at 8mm Hg).

GC-MS analysis confirms the product to be pure with very little starting material and impurities present.

Yield = 103.65g

20 m/z (GC-MS) 137 (M⁺)

* This was provided by heating a solution of 70% monoethylamine in water.

Step 2.**25 Reaction with methyl acrylate.**

N-Ethyl-m-aminophenol (48g, 0.035 mol), methyl acrylate (100ml) and acetic acid (150ml) were heated under reflux for 4 hours, after

- 23 -

which time tlc analysis (silica; dichloromethane-acetone 95:5) confirmed the completion of the reaction. The excess methyl acrylate/ acetic acid was removed under vacuum at a temperature no greater than 70°C.

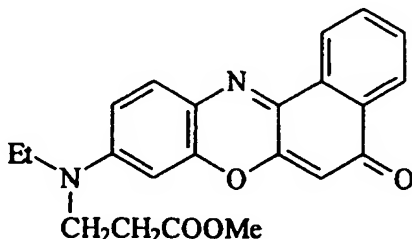
The residual oil was then neutralised with NaOH solution, extracted with
5 DCM and isolated..

Yield = 45g

m/z (GC-MS) 223 (M^+), 208 ($M^+ - Me$), 150 ($M^+ - CH_2CO_2Me$)

Synthesis of dye (XVIII) methyl ester derivative

10



N-Ethyl-*m*-aminophenol (4g, 0.0179 mol) was dissolved in
15 water (6ml) and conc. HCl (10ml) and heated to 60°C, stirring vigorously.
The solution was cooled to -5°C and a solution of sodium nitrite (10ml)
added over 10 minutes (the solution was made up by mixing 5ml of a
saturated solution of NaCl in water with 5ml containing 1.5g sodium nitrite
(0.022 moles)). Another 5ml of saturated NaCl solution was added and the
20 solution stirred at 0°C for 1 hour. The supernatant liquid was decanted off
and the oily nitroso product used directly in the next stage.

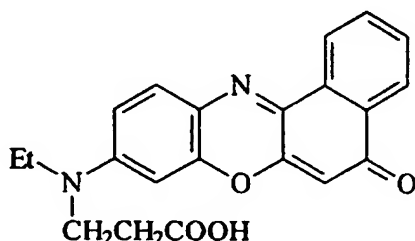
The nitroso derivative was dissolved in ethanol (40ml) to
which 1-naphthol (3.0g, 0.021 moles) was added. This solution was heated
under reflux for 20 hours after which a distinct colour change from yellow/
25 brown to violet had occurred.

The dye methyl ester was isolated by column chromatography (silica/ dichloromethane plus 5% acetone) and dried in a vacuum dessicator.

Yield = 1.00g, 15%

5 m/z (FAB) 377 (MH⁺), 399 (MNa⁺)

Hydrolysis to give dye (XVIII)



10

The methyl ester (0.5g) was stirred in water (50ml) and conc. HCl (15ml) at 80-90°C for 3 hours.

The solution was neutralised to pH 5 with 30% NaOH and the solid product filtered off and dried in a vacuum dessicator.

15 Yield = 0.34g; purity by tlc satisfactory.

UV λ_{max} (MeOH) 552 nm, $\lambda_{\text{emission}}$ (562 nm excitation) (MeOH) 636 nm.; (10mM HEPES pH 8.1) $\lambda_{\text{emission}}$ (590 nm excitation) (10mM HEPES pH 8.1) 661 nm.

20 δ_{H} (300MHz; DMSO-d₆) 1.13 (3H, t, *J* 7.0 Hz, Me), 2.57 (2H, t, *J* 7.0 Hz, CH₂CO₂H), 3.51 (2H, br. q, CH₂Me), 3.68 (2H, t, *J* 7.0 Hz, CH₂CH₂), 6.29 (1H, s, 6-CH), 6.68 (1H, d, *J* 2.6 Hz, 8-CH), 6.83 (1H, dd, *J* 9.2 and 2.6 Hz, 10-CH), 7.62 (1H, d, *J* 9.2 Hz, 11-CH), 7.71 and 7.81 (each 1H, m, 2 and 3-CH), 8.11 and 8.54 (each 1H, d, *J* 8.1 Hz, 1 and 4-CH) ppm
m/z (MALDI TOF) 362 (M⁺)

25

Example 9**Labelling of oligonucleotides with dye (VII)**

The DNA sequence T₆ was synthesised on an ABI 381A DNA synthesiser using standard reagents and cycles. The final monomer
 5 added to the 5' terminus of the resin bound oligomer was C6 aminolink (Glen Research) then the trityl protecting group was removed under standard conditions. The dye (VII) (8.6mg, 100eq.) was dissolved in DCM (0.2ml) then diisopropylethylamine (3.5µl, 100eq.) was added followed by HBTU (7.5mg, 100eq.). The solution was then added to the DNA
 10 synthesis column by double syringe methodology, i.e. two syringes attached to either side of the DNA synthesis column were used to repeatedly pass the reaction mixture through the column, for 2h. Then the column was washed with DCM (2 x 20ml). The resin bound DNA was then cleaved from the solid support by treatment of the resin with
 15 K₂CO₃/MeOH (10% w/v) for 1h., all volatiles were then removed *in vacuo*, and the product was isolated by reverse phase HPLC (DYNAMAX C18) using the following eluant system (Figure 4).

Buffer A = 0.1M Triethylammonium acetate

Buffer B = 0.1M Triethylammonium acetate / MeCN

20 (50/50 v/v)

Time / min.	Flow rate / ml/min.	% Buffer B
0	4	0
3	4	0
4	4	10
25	4	100
27	4	100
28	4	0

The isolated product was characterised by MALDI TOF MS m/z 2373.2 [$C_{92}H_{114}N_{15}O_{47}P_6$ requires 2373.8] and UV $\lambda_{max}(H_2O)$ 260 and 600 nm.

Similarly the oligonucleotide primer 5'-C6-amino-link-
5 tgtaaaacgacggccagt - 3' was labelled using the dye NHS ester (VIII) using a solution chemistry approach.

A mixture of the amino-linked primer (10 OD units) and dye NHS ester (VIII) (0.5 mg) in DMF (60 μ l) and 0.2M carbonate buffer (55 μ l) was incubated for 16 hours at room temperature. The oligonucleotide was
10 then ethanol precipitated twice and redissolved in 5% aqueous acetonitrile to give the required labelled oligonucleotide along with some unlabelled material. UV λ_{max} 260 and 598 nm

Example 10

15 **Synthesis of a range of dye labelled peptide NH_2 -AYVHDAPVRSLNK-OH**

The peptide is a substrate for Interleukin Converting Enzyme (ICE) which cleaves the peptide between the DA residues.

The peptide was synthesised on a Applied Biosystems model
20 431A peptide synthesiser using standard Fmoc chemistry. At the end of the synthesis the N-terminal Fmoc group was removed, however the protected peptide was left attached to the solid support, in which form it was reacted with the N-hydroxysuccinimidyl ester of dyes (II),(XVII) and (XVIII). The labelled peptide was then cleaved from the solid support using
25 standard techniques and then purified by reverse phase HPLC.

Synthesis of N-hydroxysuccinimidyl ester of dye (XVII).

Dye (XVII) (4mg, 7.4 μ g), N-hydroxysuccinimide (5mg, 44 μ g),
N-cyclohexyl-N'-(2-morpholinoethyl)-carbodiimide methyl p-toluene
30 sulphonate (9.3mg, 22 μ g), 4-dimethylaminopyridine (catalytic amount-one

- 27 -

small crystal) were placed in a round bottomed flask to which was added 1 ml of dry DMF. The mixture was stirred magnetically overnight (18 hrs) with light excluded at 22° C.

5 Labelling of the peptide.

32mg of the ICE substrate peptide (equivalent to 8µm peptide) was weighed into a 1.5 ml screw top polypropylene V-vial to which was added the NHS reaction mixture followed by 20µl of diisopropylethylamine. The vial was placed on rollers with light excluded for
10 20 hrs at ambient temperature (22° C). The resin was then filtered off using a sintered glass frit, washed with 5ml dry DMF, 5 ml methanol and finally 5ml dichloromethane then dried *in vacuo* for 2 hrs.

Cleavage of the labelled peptide from solid support.

15 The resin was placed in a small round bottomed flask to which was added 2ml of an ice-cold solution of trifluoroacetic acid (1.8ml), water (50µl), ethanedithiol (50µl) and thioanisole (100µl). The mixture was stirred magnetically for 90 minutes and allowed to warm to ambient temperature. The mixture was then filtered through a glass wool
20 plug and allowed to drip into 10 ml of ice cold diethyl ether. The dark blue precipitate was spun down, the supernatant removed, the precipitate redissolved in 1ml trifluoroacetic acid and reprecipitated in 10ml ice cold ether. The precipitate was spun down, washed twice with ether then dried *in vacuo*.

25

HPLC purification

The crude labelled peptide was dissolved in 1ml water, filtered through a 0.45µm Millipore filter and purified on a semi-prep. C-18 Vydac column (code 218TP510) using a gradient of 0.1% TFA/water to

- 28 -

70% of 0.1%TFA/ acetonitrile over 20 minutes and a flow of 4ml/minute.
Detection was at 230nm.

Two major peaks were eluted, the first at 10.5 minutes was colourless, the second at 13.5 minutes was blue. This second peak was
5 freeze dried to give 2.3 mg (1.15 μ m) of a bright blue solid. Mass spectroscopy of this material gave a single peak at 1995 m.u. (calculated molecular wt. of dye (XVII) labelled peptide = 1991.3)

The following dyes were also used to label the above manner using analogous procedures

10

Dye (II)

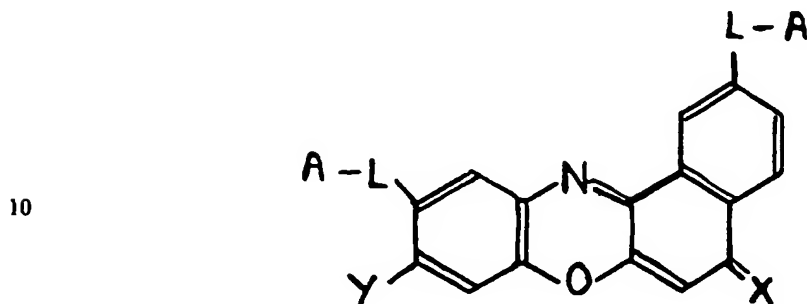
Dye (II) (3 mg) gave, after purification and freeze drying, 1 mg (0.6 μ m) of the labelled peptide as a bright blue solid. Mass spectroscopy of this material gave a single peak at 1820 m.u. (calculated
15 molecular wt. of dye (II) labelled peptide = 1812.7)

Dye (XVIII)

Dye (XVIII) gave, after purification and freeze drying, 6 mg (3.3 μ m) of the labelled peptide as a bright blue solid. Mass spectroscopy of
20 this material gave a single peak at 1816 m.u. (calculated molecular wt. of dye (XVIII) labelled peptide = 1812.7)

CLAIMS

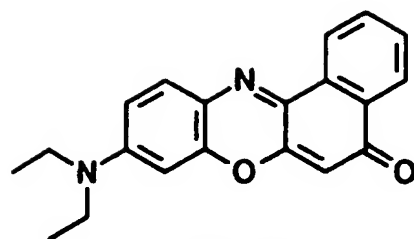
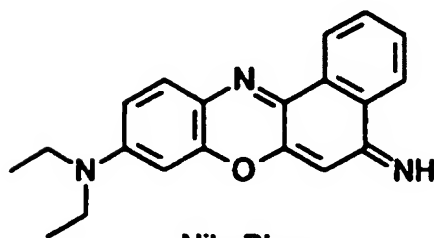
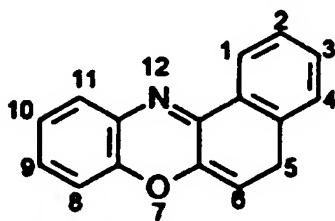
- 5 1. A benzophenoxazine compound having the formula



- 15 where X is O or NH or N-alkyl or N-aryl or N-alkenyl,
Y is -NR¹R² or H,
R¹ and R² are the same or different and each is C1 - C12 aryl,
alkenyl or alkyl or is -L-A,
in at least one -L-A:
- each L is a linker chain of 0 - 20 carbon atoms and which
20 may contain one or more O or N or S atoms, and
each A may be amine or amide or -CN or alcohol or thiol or
carboxyl or sulfonate or phosphate, or a reactive group by means of which
the benzophenoxazine compound may be covalently linked to a
biomolecule, or a group which enhances or reduces water solubility or
25 provides electron donor or withdrawal properties to modify the spectral
characteristics of the dye,
and each other -L-A is H or C1 - C20 alkyl.
2. A compound as claimed in claim 1 wherein the 2-substituent
L is -OC_nH_{2n}- or -OCOC_nH_{2n}- where n is 1 to 20.

3. A compound as claimed in claim 1, wherein in the 2-substituent L is $-\text{OC}_5\text{H}_{10}-$ or $-\text{OCOC}_4\text{H}_8-$.
4. A compound as claimed in claim 2, wherein the -L-A- group at the 2 position is $-\text{OC}_n\text{H}_{2n}\text{COOH}$ where n is 1 to 20.
- 5 5. A compound as claimed in claim 4, wherein the -L-A group at the 2-position is $-\text{OC}_5\text{H}_{10}\text{COOH}$.
6. A compound as claimed in any one of claims 1 to 5, wherein at least one group R^1 or R^2 is $-\text{C}_n\text{H}_{2n}\text{SO}_3\text{H}$ where n is 1 - 12.
7. 6-(9-N-Ethyl-(3-sulfonylpropyl)amino-5-oxo-5H-
10 benzo[a]phenoxazin-2-yloxy) hexanoic acid.
8. A compound as claimed in any one of claims 1 to 5, wherein R^1 is $-\text{C}_n\text{H}_{2n}\text{COOH}$ where n is 1-12.
9. 9-(N-2-carboxyethyl-N-ethyl)amino-5H-benzo[a]phenoxazin-5-one.
- 15 10. A complex of a compound according to any one of claims 1 to 9 and a biomolecule, wherein the compound is covalently linked to the biomolecule through a reactive group A.
11. A complex as claimed in claim 10, wherein the biomolecule is a nucleoside or nucleotide or analogue thereof, or an oligonucleotide or
20 nucleic acid.
12. A complex as claimed in claim 10, wherein the biomolecule is a protein, peptide or amino acid.
13. A complex as claimed in claim 10, wherein the biomolecule is a polysaccharide, oligosaccharide or monosaccharide.
- 25 14. A complex as claimed in claim 10, wherein the biomolecule is a drug or other small analyte.

1/5

**Nile Red****Nile Blue****Benzophenoxazine ring numbering scheme****Figure 1 Benzophenoxazine structures**

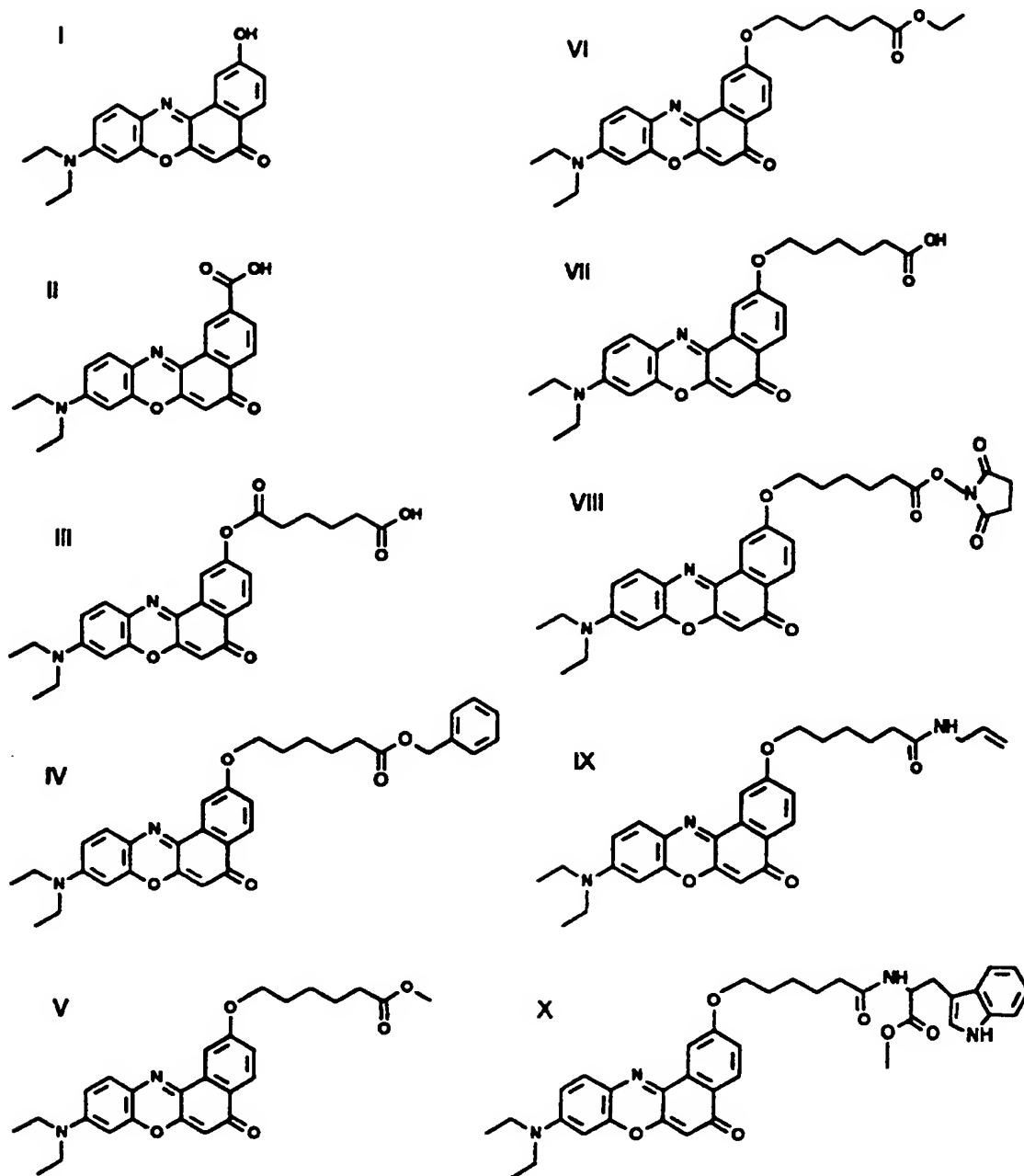


Figure 2a Structures from experimental section

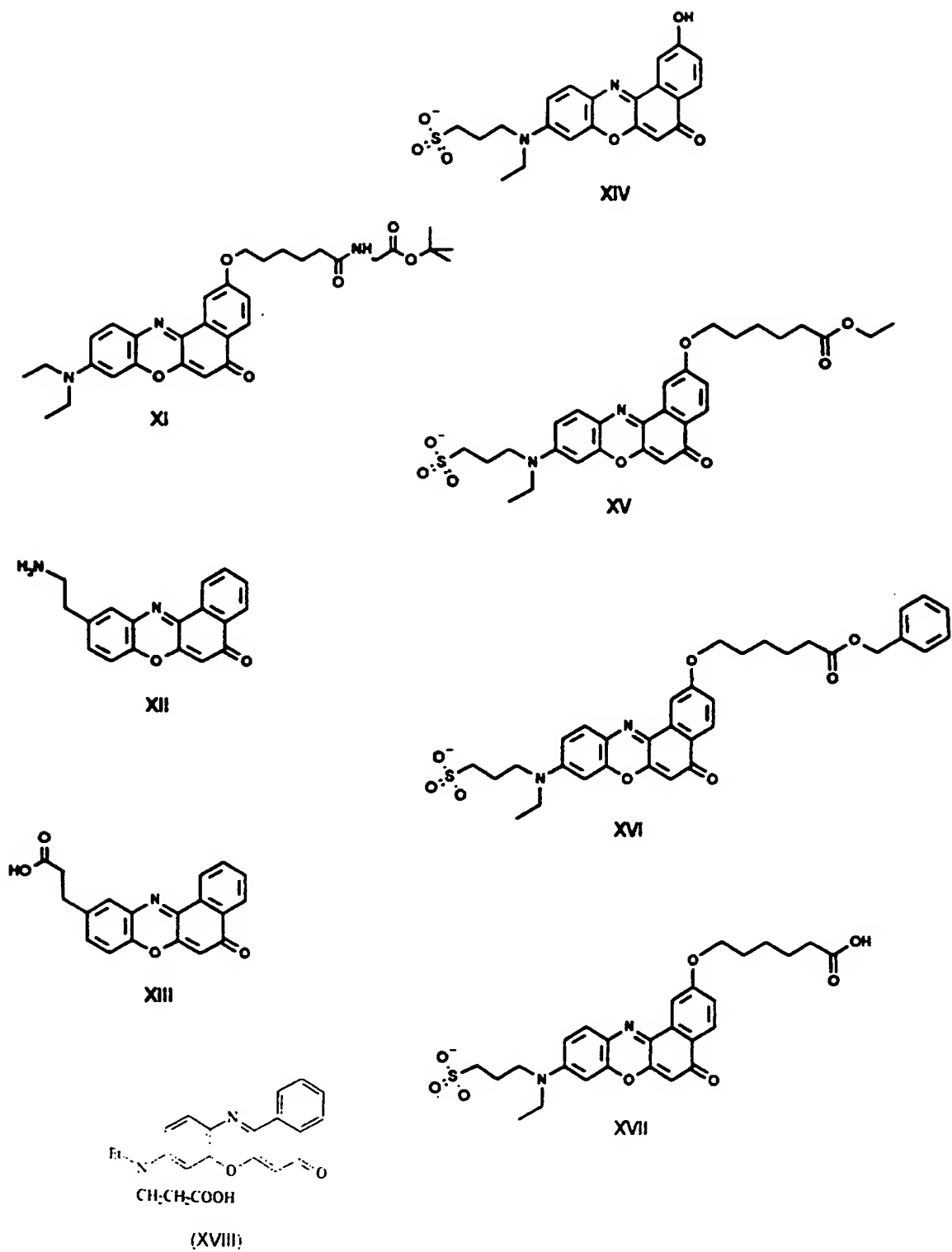


Figure 2b Structures from experimental section

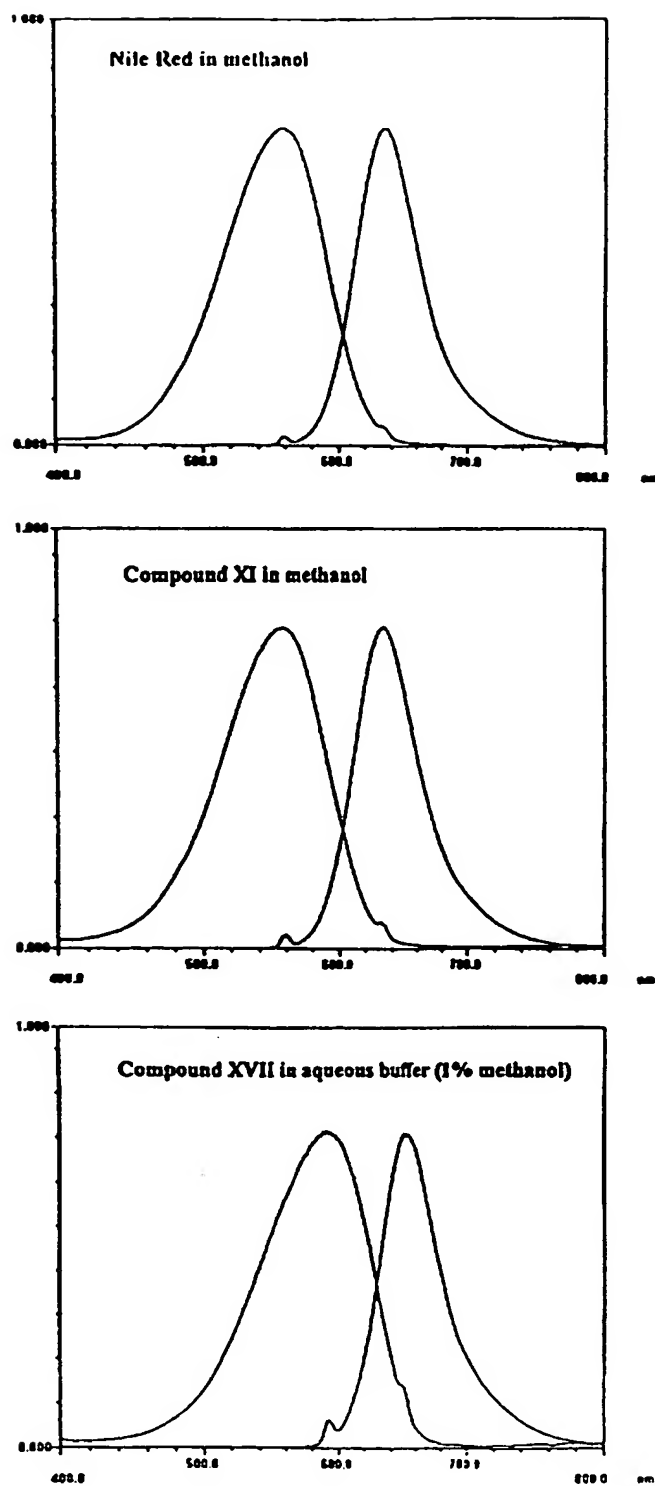


Figure 3 Normalised fluorescence spectra of Nile Red, Compound XI and Compound XVII

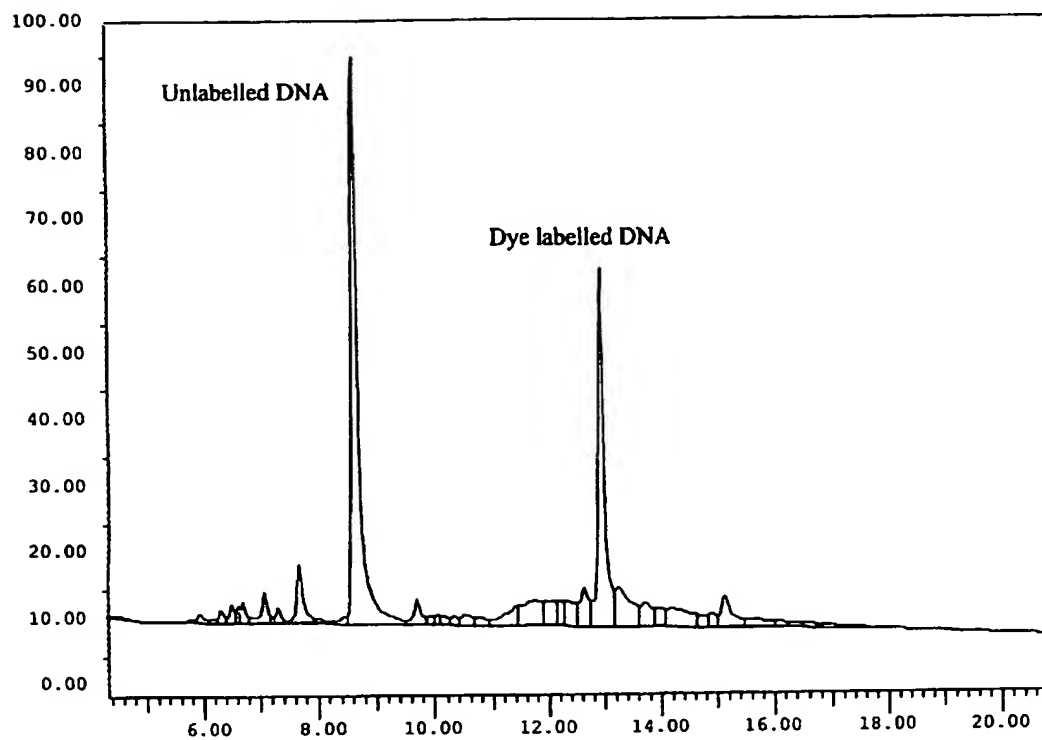


Figure 4. HPLC Analysis of Dye Labelled DNA

INTERNATIONAL SEARCH REPORT

Interr. al Application No
PCT/GB 97/00324

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C09B19/00 C07D265/34 G01N33/533

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C09B C07D G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FR 2 707 658 A (PROLABO) 20 January 1995 see page 1, line 26 - page 2, line 34 see page 4, line 26 - line 27 ---	1-14
Y	EP 0 608 737 A (BAYER AG) 3 August 1994 see abstract; examples ---	1-14
A	US 4 600 775 A (THEODOROPULOS SPYROS) 15 July 1986 see abstract ---	1-14
A	US 4 962 197 A (FOLEY JAMES W ET AL) 9 October 1990 see abstract column 2 ,compound 4 --- -/--	1-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

29.04.97

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
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Dauksch, H

INTERNATIONAL SEARCH REPORT

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	--- CHEMICAL ABSTRACTS, vol. 101, no. 25, 17 December 1984 Columbus, Ohio, US; abstract no. 230551p, ALEKSEEV, N.N. ET AL: "9-(dimethylamino)benzo(a)phenoxazin-5-one or its derivatives" page 774; XP002029604 see abstract & SU 1 109 393 A 23 August 1984	1
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Information on patent family members

International Application No

PCT/GB 97/00324

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US 4962197 A	09-10-90	NONE	
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